Limitation and detection of bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP) from bioprocess container materials

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Limitation and Detection of bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP) in bioprocess container materials

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Isabelle Uettwiller – Sartorius Stedim FMT SAS
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2  Film development and Process control
3  Sartorius Stedim experiments on different films
Interest of antioxidants in film material?

- Polymer Stabilizers such as organophosphite and stearically hindered phenols compounds are widely used to protect plastics from degradation by peroxide species
  - During the extrusion process (high temperature)
  - During sterilization (irradiation) due to chain scission or crosslinking
  - During the shelf life of the material
- Removal of antioxidants could lead to poor film properties
- TBPP: tris(2,4-di-tert-butylphenyl)phosphite (trend name: Irgafos 168) is a well-known organophosphite stabilizer described in Pharmacopeias

Single Use Bioprocess containers are used in Bioreactors for media storage & cell growth applications
bDtBPP origin (2/6)
Degradation of organophosphite stabilizer

- **Step 1:** A large fraction of TBPP is converted into oxidized TBPP during the film **extrusion process**, the remaining TBPP is converted into oxidized TBPP during the sterilization process.

- **Step 2:** After **irradiation**, further chemical breakdown occurs with the formation of bDtBPP and DtBP (2,4-di-tert-butylphenol) + other potential compounds.

(a) tris(2,4-di-tert-butylphenyl)phosphite: TBPP (ex. of Trend name: Irgafos 168)
(b) Oxidized TBPP
(c) bis(2,4-di-tert-butylphenyl)phosphate: bDtBPP
bDtBPP origin (3/6)
Degradation product of antioxidant causing detrimental effect on cell growth

Since 2013, several publications cover this subject ...
The leached compound is antioxidant-related

Leachable/extractables experiments turn up a list of compounds related to anti-oxidants...

Most used antioxidants

...one of which ("bDtBPP") severely impacts cell growth even at very low concentration

bDtBPP = Bis-(2,4-di-tert-butylphenyl) phosphate

Partnership approach to control SU variability for sustainable use in biopharmaceuticals production, Sally Kline, Magali Barbaroux, BPI Boston, October 22, 2014

VCD: Viable cell density
Impact on cell growth detected for bDtBPP concentrations in the range of 0.04 – 0.05 µg/mL

Bag suppliers send bags to the Zurich University of Applied Sciences (ZHAW)

Bags are subjected to media extractions and WFI extractions (analogue to experiments describes herein). Positive control: borosilicate glass

11 films including negative control from different suppliers

Sartorius Stedim supplied S71 (EVA) and S80 (PE) films

4 users (3 industry, 1 academia)

8 different cell lines

7 different CD media

*Eibl et al: Standardized cell culture test for the early identification of critical films for CHO cell lines in chemically defined culture media Dechema: ISBN: 978-3-89746-149-9
1. bDtBPP origin and impact on cell growth
2. Film development and Process control
3. Sartorius Stedim experiments on different films
Film is the Single Use (SU) component with the highest contact to surface ratio and highest contact time and therefore considered as the most critical one.
Film formulation answers technical and strategic needs

- Selection of basic type of polymer and molecular architecture according to the application
  - Physical properties: flexibility – robustness – gas barrier...
  - Compliance with Pharmacopoeias, Reach, TSE/BSE free
- Optimize additives in the formulation while keeping long term performances and resistance to gamma
  - Additive optimization; primary (long-term) and secondary (short-term) antiox. package, slipping agent removal...
  - Additives selection specified in Pharmacopoeias to ease tox. assessment
- Approved supplier and “block buster” polymer
  - Access to resin polymer formulation and additives by CAS number
  - Assurance of supply and change control are key factors
Film manufacturing process plays a key role in film quality

- Selection of film manufacturing process (cast versus blown extruder)
- Avoidance of water cooling to reduce endotoxin risks
- Removal of slipping agents and usage of mechanical rather than chemical antiblocking agents
- Potential release of bDtBPP reduced by different actions:
  - By reducing the quantity of TBPP (Irgafos 168)
  - By decreasing the oxidation effect: Process optimization
  - By using or increasing the quantity of other antioxidants
Film manufacturing process Design of Experiment (DOE)

**DOE critical film extrusion process parameters**

- 3 orthogonal parameters linked to residence time e.g. most critical
  - Melting $T^\circ$
  - Cooling $T^\circ$
  - Extrusion Speed (output)

**DOE Plan**

- Full factorial $2^3$ experiment
  - 8 variations
  - 3 center points

**DOE response attributes**

- Mechanical properties
- Cell growth assay
- Extractables (bDtBPP)
Evaluation of quantity of bDtBPP and Cell growth experiment

Film extrusion critical process parameter variations within design space do not impact cell growth performance

1. Amount of bDtBPP un-quantifiable in AMGEN developed extractable assay\(^1\)
   (Performed by Amgen)

2. Cell growth comparable to glass ref. throughout design space in SSB developed cell growth assay\(^2\)


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1. **bDtBPP origin and impact on cell growth**
2. **Film development and Process control**
3. **Sartorius Stedim experiments on different films**
Gamma-irradiated bags were filled with ethanol and incubated in following conditions:

**Solvent:** 100% ethanol (worst case extraction)

**Surface to volume ratio:** 1,5 cm²/mL

**Temperature:** 40°C

**Sterilisation status:** gamma-irradiation at 25-45kGy (routine dose)

**Extraction time:** 3, 21, 70, 120 days in static mode

**Film materials:** 9 different films with 2 from Sartorius Stedim are tested
An in-house SSB analytical method was developed to quantify bDtBPP based on HPLC-UV.

- **Column:** Nucleosil C18
- **Gradient:**
  - A: Acetonitrile
  - B: Water
- **Flow rate:** 1 ml/min
- **Analysis time:** 65 min
- **Temperature:** 40°C
- **Injection volume:** 20 µl

**Detection:** UV/Vis DAD-Detector wavelength 220 nm
Quantification of bDtBPP (3/6)

LOQ/LOD method validation by HPLC-UV

<table>
<thead>
<tr>
<th>5</th>
<th>0.50</th>
<th>21.966</th>
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<tbody>
<tr>
<td>6</td>
<td>0.60</td>
<td>27.508</td>
</tr>
<tr>
<td>7</td>
<td>0.70</td>
<td>32.389</td>
</tr>
<tr>
<td>8</td>
<td>0.80</td>
<td>36.4928</td>
</tr>
<tr>
<td>9</td>
<td>0.90</td>
<td>41.313</td>
</tr>
<tr>
<td>10</td>
<td>1.00</td>
<td>45.626</td>
</tr>
</tbody>
</table>

**Characteristics**

- Slope \(a\): 46.315
- Intercept \(b\): -0.440
- Correlation coefficient \(r\): 0.9996
- Result uncertainty: 33.33 %
- Probability of error (alpha): 1.00 %

**Analytical limits according to DIN 32645**

<table>
<thead>
<tr>
<th>Limit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of detection</td>
<td>0.033  mg/L</td>
</tr>
<tr>
<td>Limit of quantitation</td>
<td>0.110  mg/L</td>
</tr>
<tr>
<td>(approximation)</td>
<td>0.110  mg/L</td>
</tr>
<tr>
<td>(exact)</td>
<td></td>
</tr>
</tbody>
</table>

Number of measurements \(n\): 1

Standard error of estimate \(S_y\): 0.436

Standard error of procedure \(S_x\): 0.009

Sum of squared deviations: 0.825

Quantile (one-sided): 2.896

Quantile (two-sided): 3.355

LOD/LOQ method validation according to DIN 32645 (equivalent ISO11843-2)
Quantification of bDtBPP (4/6)
Limit of Detection and Limit of Quantification validation

- LOD and LOQ validated according to DIN 32645
- At the time of the experiment the LOD is 0.03 µg/mL and the LOQ is 0.11 µg/mL
- LOD and LOQ allow to detect the lowest quantity of bDtBPP that can impact cell growth (i.e. between 0.04 – 0.05 µg/mL\(^1\))
- Due to limited analytical experience with quantitation of bDtBPP, it has been decided to apply a LOD of 0.05 µg/mL and a reporting limit of 0.3 µg/mL

Chemical tests of bDtBPP (5/6)
bDtBPP concentrations detected in ethanol extracts from bags are close to LOD

Results show that bDtBPP level is close to the LOD in ethanol extracts which represent a worse case compared to media extraction

<table>
<thead>
<tr>
<th>Film</th>
<th>bDtBPP quantification (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t=3days</td>
</tr>
<tr>
<td>Film A</td>
<td>ND</td>
</tr>
<tr>
<td>Negative ref. Film</td>
<td>1.95</td>
</tr>
</tbody>
</table>

ND: Not detected

LOD=0.03µg/ml
Chemical tests of bDtBPP (6/6)
Comparison of bDtBPP levels between films from different suppliers

bDtBPP quantitation by HPLC-UV after EtOH extraction for t = 3, 21, 70, 120 days at 40°C
CHO based cell growth assay and dose response to bDtBPP established

Standardized cell growth assay

- rCHO DG44 cells grown in 6 well plates in protein free cell culture medium
- Medium incubated for 3 days, at 37°C in γ-irradiated sample bags at a volume-to-surface-ratio of 3cm²/ml
- Control: medium incubated 3 days, at 37°C in borosilicate bottle

![Graph showing cell growth and viability response to bDtBPP concentration]
Cell growth testing (2/2)
Routine cell growth testing of film batches shows consistent cell growth and therefore proves that the amount of bDtBPP is below the detrimental level.

[Bar chart showing normalized cell growth for different batches: Reference, I, II, III, P1, P2, P3, P4. Each batch has a different number of testing samples: N=6, N=27, N=30, N=9, N=9, N=9, N=9. The internal acceptance criteria is 90%.]
Key Take Home Messages

- The link between cell growth performance and quantity of leaching bDtBPP from plastic materials has been verified through various studies and publications.
- Analytical method (HPLC-UV) has been implemented at Sartorius Stedim to verify the quantity of bDtBPP leaching compound with an acceptable Limit of detection.
- Verification of Cell growth experiment on film material from Sartorius Stedim demonstrate very good performance of the film developed with formulation and process optimization.

Film material with reduced quantity of Phosphite antioxidant and optimized extrusion parameters can lead to both polymer degradation protection and good cell growth performance.

Variability of the film quality is limited with a strong control of your supplier on the resin formulation and the extrusion parameters.
Acknowledgements

- Magali Barbaroux
- Samuel Dorey
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- Thomas Loewe
- Ina Pahl
- Roberto Menzel

And our external partners........
Thank you very much for your attention!